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14. ABSTRACT
Breast cancer is the most common type of cancer and a leading cause of death among Western women. Most breast cancers (75%) express ERα, and antiestrogens have been widely used in their treatment. However, acquired resistance and unwanted side effects in other estrogen-responsive tissue such as uterus have greatly limited their use. The objective of this study to isolate RNA aptamers that specifically target the estrogen receptor interacting NR boxes/LxxLL motifs of the MED1 protein and test their efficacy on breast cancer cell growth both in vitro and in vivo by utilizing a pRNA nanodelivery system for tissue-selective therapy. In this funding period, we have further tested and optimized the RNA aptamers obtained by SELEX method, based on its structure and ability to inhibit breast cancer cell growth, migration and estrogen-dependent gene expression. Through these studies, we have obtained one most promising RNA aptamer that can specifically disrupt the ER/MED1 interaction. Importantly, this aptamer not only greatly inhibited breast cancer growth, migration and ER target gene expression in vitro, but also blocked tumor growth in an orthotopic xenograft mouse model.

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RNA Aptamer, SELEX

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Introduction

Breast cancer is the most common type of cancer and a leading cause of death among Western women. Most breast cancers (75%) express ERα, and antiestrogens have been widely used in their treatment. However, acquired resistance and unwanted side effects in other estrogen-responsive tissue such as the uterus have greatly limited their use. This study is based on our recent unexpected finding that disruption of the transcriptional coactivator MED1 nuclear receptor-interacting NR boxes/LxxLL motifs in vivo specifically impaired ERα function in pubertal mammary gland development, but did not affect the development of other estrogen-responsive tissues. Significantly, MED1 is reported to be overexpressed and amplified in a high proportion of primary breast cancers and breast cancer cell lines, which has recently been confirmed in several genome-wide microarray analyses of human breast cancer patient samples. We *hypothesize* that targeting MED1 NR boxes /LxxLL motifs by aptamers will lead to tissue-selective blockage of the estrogen signaling pathway and inhibition of human breast cancer cell growth. The objective of this study is to isolate RNA aptamers that specifically bind the MED1 LxxLL motifs and test their efficacy on breast cancer cell growth both in vitro and in vivo by utilizing a pRNA nanodelivery system.

Body

To achieve our above-mentioned goals, we have generated a MED1 NR boxes expression vector and subsequently purified wild type and mutant MED1 NR boxes /LxxLL motifs protein. In addition, we have also constructed an RNA aptamer library and worked through the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) procedure to isolate RNA aptamers that bind to MED1 NR boxes/LxxLL motifs. We have successfully carried out 6 rounds of SELEX procedures and obtained several RNA Aptamer candidates that interact with MED1 NR boxes during the last funding period. In this funding period, we have performed further experiments to select the best RNA aptamer candidate, optimized its sequence and tested its functions on ER-mediated functions and breast cancer cell growth both in vitro and in vivo.

Functional selection of MED1 aptamers by its ability to impair estrogen-dependent transcription, cell growth and migration

During this funding period, we have successfully completed the SELEX procedures and selected 11 top candidates based on their highest percentage of presence out of total sample and highest frequency between multiple SELEX rounds. We further tested their ability to inhibit the growth of breast cancer cells by using an MTT assay after transfecting them into MCF-7 cells with lipofectamine. We found that 6 of these aptamers (R, P, T, O, K, X) are able to significantly inhibit the growth of MCF-7 cells when compared to that of no treatment or control with scramble (S) RNA sequences of the same length (Figure 1A). To further determine whether these aptamers affect the estrogen-dependent gene transcription, we carried out ERE-luciferase reporter gene assays for each of these aptamers (Figure 1B). We found that aptamers R, P, T, O and X significantly inhibited the transcriptional level of ERE-luciferase gene expression in MCF-7 cell lines when compared to a scramble control aptamer. Next, we carried out transwell cell migration assays and found that all of these RNA aptamers can significantly inhibit the cell migration capacity (Figure 1C-D) with some of the aptamers showing as high as 75% inhibition.

These results indicate that the aptamers we isolated are capable of blocking estrogen-dependent transcription, cell growth and migration of breast cancer cells.

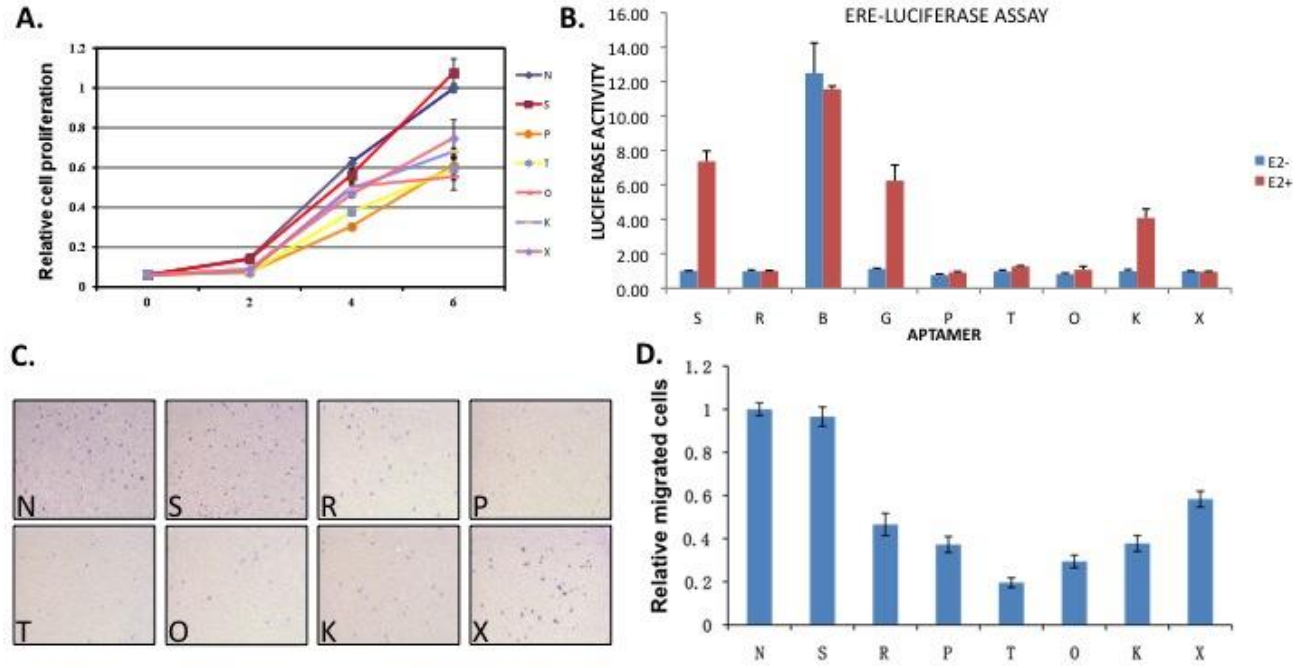

Figure 1. MED1 RNA Aptamers impair Estrogen-Dependent Transcription, Cell Growth and Migration. A) Effect of indicated RNA aptamers on the proliferation of MCF-7 cells by MTT assay. B) Luciferase Assay conducted after transient transfection of indicated RNA aptamers with ERE-luciferase reporters. C) Transwell migration assay of MCF-7 cells after transfection with the indicated control and isolated MED1 RNA aptamers. D) Quantitation of transwell assay results in (C).

Optimization of RNA aptamers based on structural predication and functional assays.

Since these aptamers have a length of 80nt, we decided to further identify the minimal region of the RNA aptamers required for its inhibition of ER-dependent transcription, breast cancer growth and migration. Identification of the shortest sequences required could provide advantages for better incorporation into a pRNA nanodelivery system and will be more cost effective for large scale production for future preclinical and clinical studies. Interestingly, based on mFOLD analyses of secondary structure of these isolated RNA aptamers, we found that they all contain a Loop-Stem-Loop-Stem-Loop-like structure with slightly different size of loop or length of the stems (Fig. 2A). Therefore, we decided to first make deletions based on this information to better understand their structure-function relationships and identify the minimal sequence required for function. We designed new sets of DNA oligos for each aptamer of interest and generated short versions of RNA aptamers by RNA transcription. Upon subjecting these shortened aptamers to the transcriptional and migration assays as done for their full-length versions, we found short aptamer (S indicating short henceforth) SP showed most significant ability in disrupting ER-dependent transcription (Figure 2B) and migration (Figure 2C-D) of breast cancer cells. Based on these data, we decided to focus our future experiments on the SP RNA apamter for further studies as described below.

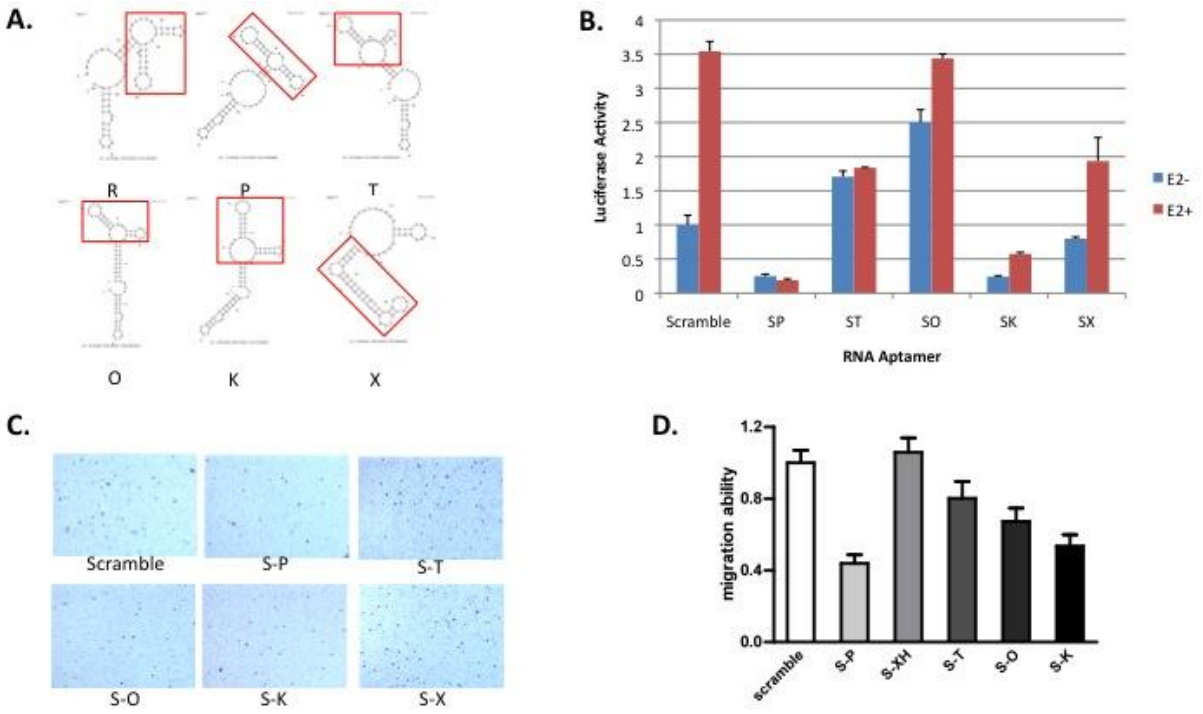

Figure 2. Shortened Aptamers Retain Their Ability to Block Breast Cancer Cell Migration and Estrogen-Responsive Gene Transcription.

A) Structural analyses of isolated RNA aptamers by mFold software. Regions outlined by red box indicate common loop-stem-loop-stem-loop structure in all aptamers. B) ERE-luciferase reporter assay with the short version of the RNA aptamers. C) Transwell migration assay of the indicated control scramble and shortened RNA aptamers. D) Quantitation of migration assay in (C).

SP aptamer specifically blocks ER/MED1 interaction and ER-dependent gene transcription.

As shown in Fig. 3A, we found that the SP aptamer can block ER-dependent gene transcription as robustly as the original P aptamer by the above-mentioned ERE-luciferase reporter assays. To further determine the underlying molecular mechanism and test whether this blocked ER-dependent transcription activity was due to the ability of the SP aptamer to block the ER/MED1 interaction, we carried out GST pulldown assays. In this experiment, we purified GST-ER proteins and mixed with Hela nuclear extract (NE) in the presence of 1 microgram (ug) of scramble aptamer or the indicated different amounts of the SP aptamer. After rotating for 3-4 hours and extensive wash, the bound proteins were subjected to SDS-PAGE and blotted with antibodies against MED1. We found that when a gradient of concentrations (0.2 ug, 0.5 ug, 1 ug) of the short P aptamer were applied, there was a clear increase in inhibition of ER/MED1 interaction with the increased amount of the SP aptamer (Figure 3B). Importantly, this inhibition of ER/MED1 interaction by SP aptamer is specific because the presence of no aptamer or the scramble control aptamer does not affect the interaction between ER and MED1 (Figure 3C). Moreover, the interaction between ER and another ER coactivator SRC-1, which contains similar but different ER interacting LxxLL motifs, was not affected by SP aptamer or controls (Figure 3C). These data indicate the strong specificity of the SP aptamer in disrupting ER/MED1 interaction and support our further *in vitro* and *in vivo* testing.

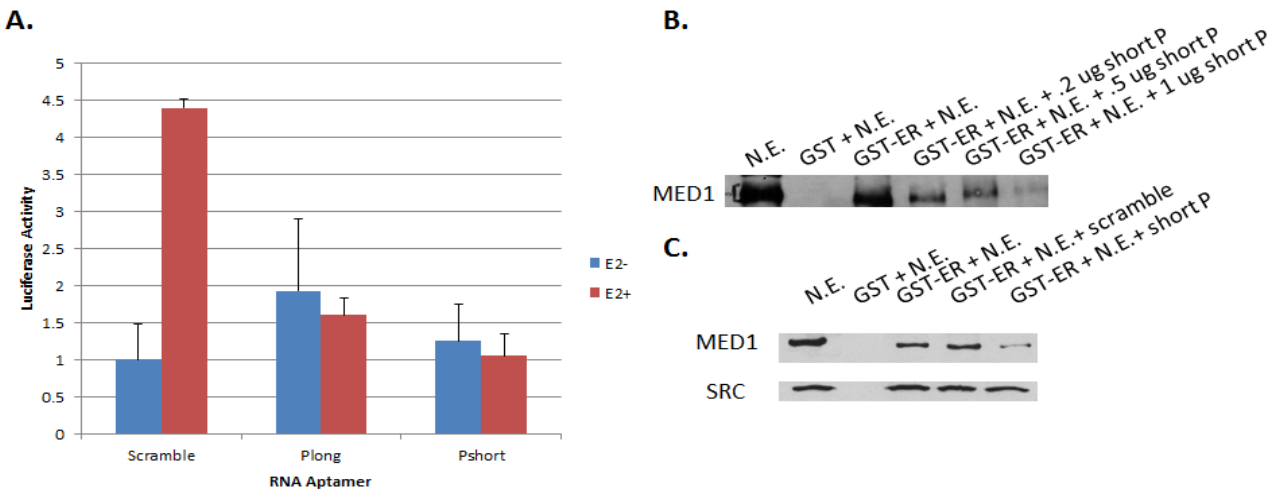

Figure 3. Shortened "P" Aptamer Specifically Inhibit ER/MED1 Interaction and ER-dependent Transcription

A) ERE-luciferase reporter assay was performed as in figure 1 comparing the original and short versions of the P aptamer. B) GST-pulldown assay using GST-ER and Hela nuclear extract (NE) with increasing concentrations of the SP aptamer. C) GST-pulldown assays using GST-ER and Hela nuclear extract with 1ug of control scramble and SP aptamer. Together, these results indicate SP aptamer can block the estrogen-dependent transcription in MCF-7 breast cancer cells and disrupt the interaction of ER/MED1 but not the interaction between ER and another coactivator SRC-1.

Folate conjugated SP aptamer inhibits breast cancer cell growth both in vitro and in vivo in orthotopic xenograft mouse model.

Above, we have identified the SP aptamer and tested its function in breast cancer cells in estrogen-dependent transcription, growth and migration using a lipofectamine-mediated method. To facilitate our further in vitro and in vivo testings, we synthesized an SP aptamer with Folate attached at the 5' end for specific targeting and uptake by breast cancer cells. In addition, we added a fluorescent label (Alexa Fluor® 647) at the 3' end for in vivo imaging purpose. Importantly, folate receptors have been found to be highly expressed in a group of cancer cells and folate has been successfully exploited to introduce folate-conjugated macromolecules into these cancer cells. We first tested the folate-SP aptamer on the growth of ER positive human breast cancer cells, BT474, which are known to express high levels of folate receptor. We added the indicated amount of folate-SP aptamer directly to the cell culture medium and performed cell proliferation MTT assays (Figure 4A). We found an approximate 40% reduction in cell growth using concentrations of 50-100 ug/ml of the aptamer. The migration of these cells was further tested and the data indicate that SP strongly inhibited migration ability of BT474 cells (Fig. 4B). Moreover, we performed realtime RT-PCR analyses after isolating the total RNA from the control and folate-SP aptamer treated cells. We found that the expression of well-known ER target genes C-Myc, TFF1 and Cyclin D1 are all significantly blocked by the folate-SP aptamer when compared to that of control. Finally, we tested the function of the folate-SP aptamer on breast cancer growth in vivo using an orthotopic xenograft mouse model. 1×10^6 BT474-Luc cells were suspended in 50% Matrigel (BD Bioscience) at the final volume of 100 µl and injected into the fat pad of the fourth glands of 7-week-old female NOD-SCID mice. When tumors reached the size of ~50 mm^3, the mice were further randomly distributed into 2 groups and treated with the control or folate-SP aptamer. We found that folate-SP aptamers were

specifically targeted to the tumor region 96 hrs post-injection as monitored with an IVIS® Lumina Living Image System (Figure 4D). Tumor progression was further monitored using the same Lumina Living Image System after injection of luciferin to detect the BT474-luc breast cancer cells. As shown in Figure 4E-F, we found the growth of xenografted tumors was significantly impaired by the folate-SP aptamer treatments when compared to that of control treatment. Together, these results indicate that the folate-SP aptamer can be not only inhibit cell growth, migration the expression of endogenous ER target genes but also block human breast tumor growth in vivo in an orthotopic xenograft mouse model.

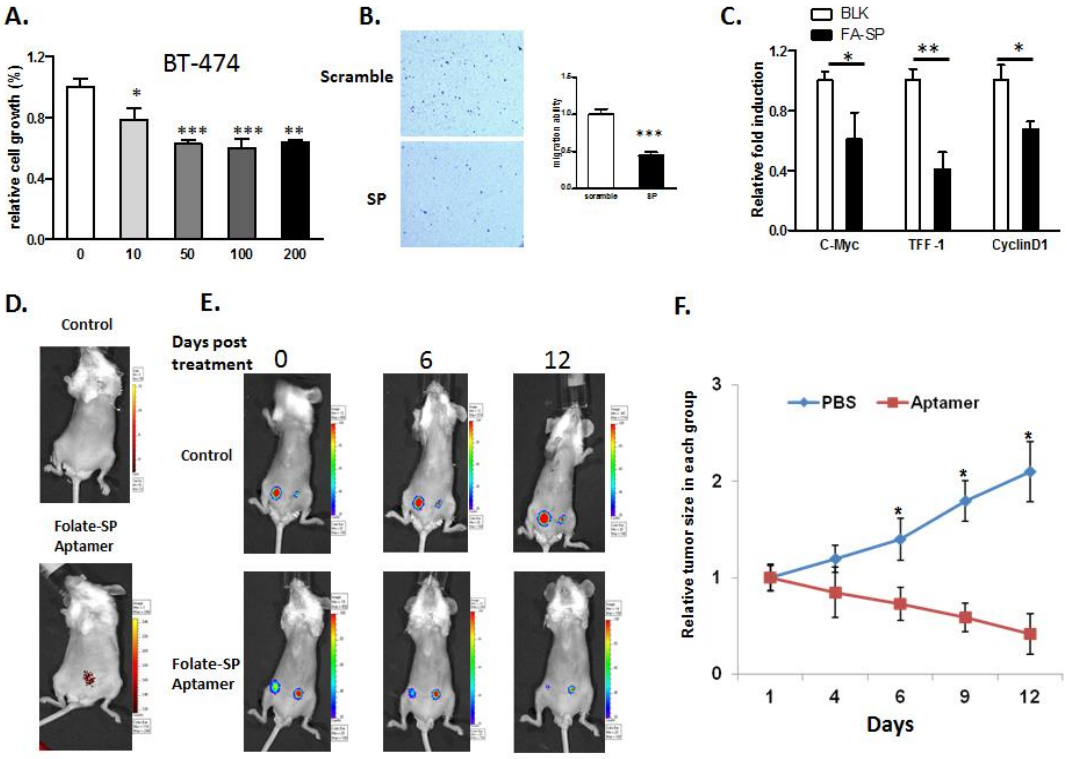

Figure 4: Targeted Delivery of SP by Folate Conjugation Inhibits Breast Cancer Cell Growth, Migration, Endogenous ER Target Gene Expression.

A) Cell proliferation MTT assays of BT474 cells after the treatment with the indicated amount (ug/ml) of folate conjugated SP RNA aptamer. B) Cell migration assay after the control (mock) and folate-SP aptamer treatment and the quantification. C) Real time PCR analyses of indicated ER downstream target genes after folate-SP treatment. D) Localization of folate-SP aptamer to the tumor 96 hrs post-injection as detected by an IVIS® Lumina Living Image System. E) Bioluminescence live images of tumor xenograft at 0, 6, 12 days post treatment of control or folate-SP aptamer. F) Quantitation of relative tumor growth based on the image obtained as in (E). Together, these results indicate folate-SP aptamer can block breast cancer cell growth both in vitro and in vivo. Statistical significance *: $p< 0.05$, **: $p< 0.01$, ***: $p<0.001$.

Key Research Accomplishments

- 11 highest ranked MED1 aptamers isolated were tested based on their ability to inhibit estrogen-dependent transcription and breast cancer cell growth
- Aptamers R, P, T, O, X show best ability in inhibition of estrogen-dependent transcription and breast cancer cell growth
- After structural analysis via mFold software, these aptamers were further optimized to minimal length while maintaining folded structure.
- These shorted RNA aptamers were further tested *in vitro* by ERE-luciferase reporter and transwell migration assays
- Shortened aptamer P shows most significant inhibition of breast cancer cell proliferation and migration, as well as the best ability of all shortened aptamers to block estrogen-dependent gene transcription.
- Mechanistic studies indicate that the shortened P aptamer can specifically block the interaction specifically between ER and MED1, but not the interaction between ER and another coactivator SRC-1.
- Conjugation of shortened P aptamers to Folate is able to target BT474 cells that express high levels of folate receptor and inhibit both cell proliferation and migration.
- Real time PCR analysis demonstrates SP aptamer is able to effectively block transcription of endogenous ER target genes including C-myc, TFF-1 and Cyclin D1.
- Folate-SP aptamer can specifically target and inhibit the growth of orthotopically implanted human breast cancer BT-474 cells in vivo.

Reportable Outcomes

In this reporting period, we have two manuscripts (Attached as Appendices for this report) published:

Papers:

Germer K, Pi M, Guo P and **Zhang X.** Conjugation of RNA aptamer to pRNA nanoparticles for RNA-based therapy. (2013) RNA Nanotechnology and Therapeutics, CRC Press 2013 Pages: 399-408 (Corresponding Author)

Leonard M, Zhang Y and **Zhang X.** Small non-coding RNAs and aptamers in diagnostics and therapeutics. (Invited Book Chapter) (2014) Methods in Molecular Biology. Springer Protocols.

Conclusion

In conclusion, during this funding period, we have tested the top 11 aptamer candidates and selected the top five aptamers based on their efficacy in inhibiting breast cancer cell growth, migration and ER-dependent gene transcription. We have further optimized five of these top 11 RNA aptamers based on structural analyses to identify the minimal functional sequence while maintaining the same folded structure and inhibitory properties. Via ERE-luciferase reporter assays and transwell migration assays, we have determined that shortened aptamer SP has the most potential in the inhibiton of breast cancer cell growth and migration, along with the greatest ability to block gene transcription of ER-target genes like C-myc, TFF-1 and Cyclin D1. Furthermore, we have shown that the SP aptamer is highly specific for the ER/MED1 interaction, as the interaction between ER and other transcriptional coactivators, like SRC-1, was not affected by the aptamer. Importantly, our studies have also indicated that this folate-SP aptamer can effectively target human breast cancer cells and inhibit cell growth in vivo in an orthotopic xenograft mouse model. Together, the data indicate that we have successfully identified a novel prototype MED1 RNA aptamer that can serve both as a tool to study the basic biology regarding the functional significance of ER/MED1 interaction and, most importantly, a promising novel therapeutic regimen that could be further tested alone, or in combination with current endocrine therapies for the treatment of human breast cancer.

References

N/A

Appendices

Paper 1: Conjugation of RNA aptamer to RNA nanoparticles for targeted drug delivery.

Paper 2: Small non-coding RNAs and aptamers in diagnostics and therapeutics.

19

Conjugation of RNA Aptamer to RNA Nanoparticles for Targeted Drug Delivery

Katherine Germer, Fengmei Pi, Peixuan Guo, and Xiaoting Zhang

CONTENTS

19.1 Introduction

RNA-based therapy has become a promising avenue for the treatment of many human diseases. The therapeutic potential of RNAs, including ribozymes, short hairpin RNA, small interfering RNA (siRNA), microRNA, antisense oligonucleotides, and RNA aptamers, has long been extensively studied (Guo 2010; Keefe et al. 2010; Levy-Nissenbaum et al. 2008; Que-Gewirth and Sullenger 2007; Yan and Levy 2009). A major challenge that remains is the systemic delivery of these moieties (siRNA, ribozyme, etc.) to the desired target cell organelles. In this regard, the packaging RNA (pRNA) nanoparticle delivery system pioneered by Dr. Guo, combined with recent advancement in RNA aptamers, provides an ideal method for nanoscale delivery suitable for in vivo targeted delivery (Guo 2010).

pRNA nanoparticles are a new RNA-based nanoparticle drug delivery system, which can be designed and constructed by phi29 pRNA through dimer formation, hexamer formation, or using its stable three-way junction (3WJ) domain as a scaffold. Because pRNA nanoparticles are composed entirely of RNA, it is the most natural choice for the delivery of RNA therapeutics because using an all-RNA delivery system will also allow all the advantages of RNAs as therapeutic agents to be retained. One key obstacle for RNA-based therapy is that RNA is susceptible to quick degradation in the bloodstream during in vivo delivery. To overcome this, researchers from Dr. Guo's laboratory have recently developed

highly stable and RNase-resistant pRNA through elaborate design of RNA sequences and chemical modifications such as 2′-deoxy-2′-fluoro (2′-F) modification at the ribose rings of C and U (Liu et al. 2011; Shu et al. 2011).

RNA aptamers are RNA oligonucleotides capable of binding to specific targets with high affinity and specificity. RNA aptamers have numerous advantages for targeted drug delivery when compared with DNA aptamers, protein aptamers, and antibodies (Guo 2010; Keefe et al. 2010; Que-Gewirth and Sullenger 2007; Thiel and Giangrande 2010). Compared with their peptide and antibody counterparts, RNA aptamers are much easier to synthesize in large quantities with defined structure and stoichiometry. Furthermore, RNA aptamers are generally considered to be more thermodynamically stable than peptides or antibodies. Although RNA aptamers function similarly to antibodies, they are known to have low or no immunogenicity when compared with other macromolecules such as proteins/antibodies. Furthermore, recent studies have found that RNA aptamers can be further chemically modified (e.g., 2′deoxy, 2′F, 2′NH3, 2′OMe) to achieve high stability and evade RNase shearing even in the bloodstream. Moreover, the single-stranded nature of RNA aptamers not only allows them to form unique tertiary structures for tighter and more specific binding to the target but also makes them smaller in size and thus allowing easier access into cells compared with other types of aptamers. Thus, the conjugation of RNA aptamers to delicately designed pRNA nanoparticles can assist in the delivery of therapeutics harboring RNA nanoparticles to specific cell organelles to maximize therapeutic effects while minimizing the toxicity of the drug delivery system.

19.2 Structure of RNA Nanoparticles

The concept of RNA nanotechnology has been proposed for more than one decade. Most RNA molecules are single-stranded nucleotides, which can adopt very complex three-dimensional structures. Therefore, RNA is an ideal biocompatible material that is suitable for construction at the nanometer scale for drug delivery, especially for oligonucleotide-based drug delivery.

19.2.1 RNA Nanoparticle Formed by Dimerization of phi29 pRNA

Phi29 pRNA is a 117-nucleotide bacteriophage phi29 encoded pRNA discovered by Dr. Guo in 1987 (Guo et al. 1987). The pRNA monomer plays an essential role in packaging DNA into procapsid by forming a hexamer ring to drive the DNA packaging motor of bacteriophage 29, which is approximately 11 nm in size. The primary structure of wild-type pRNA is described in Figure 19.1 (Liu et al. 2011). pRNA has two functional domains that can fold independently: the DNA translocation domain and the prohead binding domain. The DNA translocation domain is composed of a 3′/5′ double helix loop whereas the prohead binding domain is composed of left-hand and right-hand loops. If we name the right-hand loop with uppercase letters (A, B, C) and name the left-hand loop with lowercase letters (a, b, c) denoting different loop sequences, the RNA sequence for A, B, C are complementary to sequence a, b, c, respectively. pRNA dimer nanoparticles can be formed through the complementary hand-in-hand loop interactions between pRNA monomer Ab and Ba as described in Figure 19.2 (Shu et al. 2004). The pRNA dimer nanoparticle has been reported to have a particle size of approximately 25 nm (Chen et al. 2000), which allows it to be employed as a nanoparticle carrier for gene drug delivery because its small size allows it to escape from being engulfed by the reticuloendothelial system, and to be used for repeated and long-term gene drug delivery.

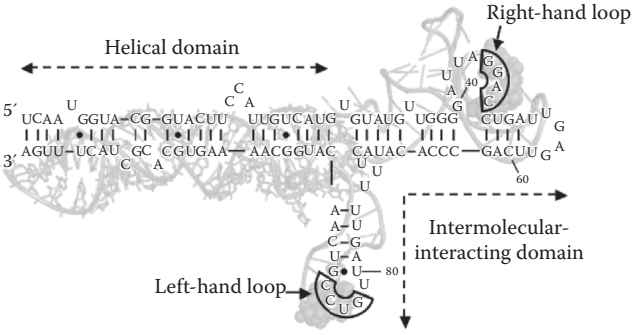

FIGURE 19.1
Primary sequence and structure of wild-type pRNA. (Reprinted with permission from Liu, J. et al., Fabrication of stable and RNase-resistant RNA nanoparticles active in gearing the nanomotors for viral DNA packaging, *ACS Nano* 5, 237–246. Copyright 2011 American Chemical Society.)

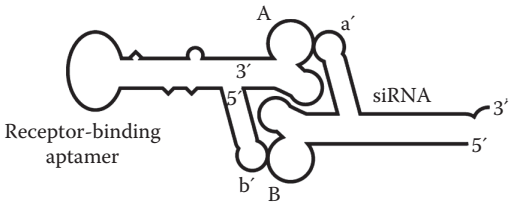

FIGURE 19.2
pRNA dimer formed through hand-in-hand complementary loop interactions. (Reprinted by permission from Macmillan Publishers Ltd. Guo, P. et al., The emerging field of RNA nanotechnology, *Nat Nanotechnol* 5, 833–842, copyright 2010.)

19.2.2 RNA Nanoparticle Composed by Using pRNA Hexamer as Scaffold

pRNA dimers are building blocks for pRNA hexamer formation, which has been proved by Chen et al. (2000), through hand-in-hand interactions between two complementary pRNAs that can form dimers, tetramers, and hexamers. A schematic drawing for the pRNA hexamer formation is described in Figure 19.3. The six pieces of pRNA in pRNA hexamer nanoparticles could provide six positions to conjugate therapeutic molecules such as siRNAs, ribozymes, therapeutic RNA/DNA aptamers, or diagnostic RNA/DNA aptamers for drug delivery.

19.2.3 RNA Nanoparticles Based on 3WJ Motif

A third strategy for constructing pRNA nanoparticle is based on a thermodynamically stable RNA 3WJ motif (Shu et al. 2011). pRNA has two functional domains that can fold independently: a DNA translocation domain and a prohead binding domain. The two domains are connected by a 3WJ motif, as described in Figure 19.4. The 3WJ domain of pRNA was demonstrated to be very stable, which can retain its folding even in 8 M of urea or at very dilute concentrations. By conjugating the RNA therapeutic molecules such as siRNA or RNA aptamers to the 3WJ motif, they can self-assemble to form RNA nanoparticles, which can potentially be used for targeted RNA therapeutic delivery in vivo.

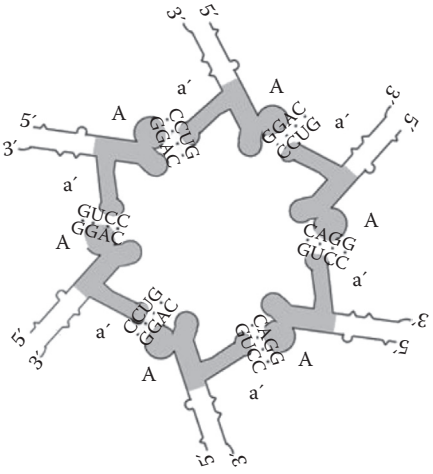

FIGURE 19.3
Schematic drawing of pRNA hexamer through complementary loop–loop interaction with six pieces of pRNA monomer. (From Chen C. et al., *J. Biol. Chem.* 275, 17510–17516, 2000. With permission from the American Society for Biochemistry and Molecular Biology.)

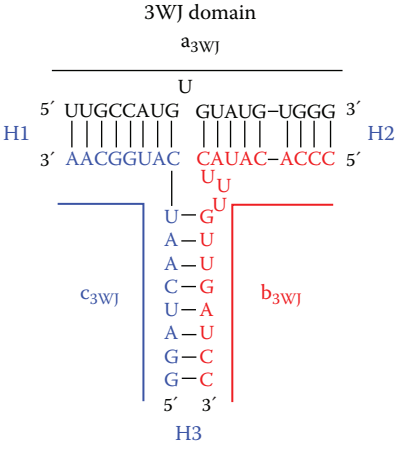

FIGURE 19.4
3WJ motif of pRNA, which can be used for RNA nanoparticle construction. (Reprinted by permission from Macmillan Publishers Ltd. *Nat. Nanotechnol.*, Shu, D. et al., Thermodynamically stable RNA three-way junction for constructing multifunctional nanoparticles for delivery of therapeutics, 6, 658-667, copyright 2011.)

19.3 Conjugate RNA Aptamer to RNA Nanoparticles for Targeted Drug Delivery

The concept of a targeted drug delivery system was proposed by Paul Ehrlich in 1902, in which he first called the hypothetical drug a "magic bullet" (Ehrlich 1957). In general, targeted drug delivery requires the targeted drug delivery system (TDDS) to selectively

deliver therapeutics to diseased regions, independent of the method of its administration. The TDDS can be classified into three grades from the aspect of the region it reaches: the first grade targeting system refers to delivering the drug to a targeted organ or tissue, the second grade targeting system refers to delivering the drug to specific cells, and the third grade targeting system refers to delivering therapeutic molecules to specific locations inside the cell.

The TDDS can also be divided into three types based on the pattern of targeting: passive, active, and physical. There has been intensive research on passive targeting drug delivery systems such as liposomes, nanoemulsions, microcapsules, and polymeric nanospheres in the last several decades. Passive targeting relies on the natural distribution pattern of the drug delivery system, as the drug carriers can be ingested by macrophages of the reticuloendothelial system and then transferred primarily to the liver and spleen. However, it is difficult to deliver drugs to other organs with the passive targeting mechanism because the in vivo distribution of the passive targeting drug carriers is greatly affected by its particle size and the surface property of nanoparticles. As a general rule, when the particle size is larger than 7 μm, it will be retained by the smallest lung capillaries through mechanical filtration; when the particle size is smaller than 7 μm, it is ingested by macrophages in the liver and spleen; carriers of particles between 200 and 400 nm are usually collected and are rapidly cleaned up by the liver. Active targeting preparations instead utilize modified drug carriers as a "bullet" to directionally concentrate drugs to the target area for enhanced efficacy. These modifications include PEGylation of the nanoparticles to conceal the particle from macrophages, conjugation with special ligands or antibodies, which can interact with the target cell receptor, and other approaches. Physical and chemical targeting preparations utilize physical or chemical properties to help navigate preparations to specific targeting locations. For example, magnetically targeted drug delivery incorporates magnetic material into the drug preparation, and the preparation will then be concentrated to the specific target area under the guidance of an externally applied magnetic field, whereas thermal or pH targeting drug delivery utilizes thermal or pH-sensitive material to deliver the therapeutics to specific macroenvironments by changes in temperature or pH.

19.3.1 RNA Aptamers and SELEX

RNA aptamers are RNA oligonucleotides that bind to a specific target with high affinity and specificity, similar to antibody interaction with antigens. RNA aptamer isolation was initially developed in two separate laboratories by Turek and Gold (1990), and by Ellington and Szostak (1990) through a process that eventually became known as systematic evolution of ligands by exponential enrichment, or SELEX. To begin the SELEX process, a library of randomized pools of RNA will first be synthesized. Generally, these RNA oligonucleotides are designed with a random sequence of nucleotides of approximately 20 to 80 nucleotides in the center region that is flanked on either side by a constant sequence. This library of oligonucleotides will then be exposed to the target of interest, which could be small molecules, proteins, cells, or even organisms (Dua et al. 2011; Keefe et al. 2010; Levy-Nissenbaum et al. 2008; Thiel and Giangrande 2010). Those that do not bind to the target are washed away and discarded, whereas those that do bind are isolated and amplified through reverse transcription and PCR to generate a corresponding DNA library. The DNA library is then subjected to RNA transcription, and the resulting RNA library will then be exposed again to the target of interest for another round of the SELEX process. This process is usually repeated about 5 to 15 times, and the aptamers obtained could often reach a high picomolar to low nanomolar range of dissociation constants (K_d) with the target (Dua et al. 2011; Yan and Levy 2009).

Through this basic SELEX process, and some recently developed variations of this process such as Cell-SELEX, Cross-over SELEX, and Tissue-SELEX, a good number of RNA aptamers have been isolated with the capability of binding numerous specific targets (Dua et al. 2011; Levy-Nissenbaum et al. 2008; Yan and Levy 2009). Significantly, many of these targets are cell surface markers of various human diseases, which has led to the application of these RNA aptamers for targeted delivery of RNA therapeutics, especially those based on RNA interferences: siRNA, short-hairpin RNA, or microRNA.

19.3.2 Approaches to Conjugate Aptamer to RNA Nanoparticle

The key step in the construction of RNA aptamer–conjugated nanoparticles is to design the global structure according to the physical and chemical properties of the RNA nanoparticles. If the RNA aptamer has been selected with a known sequence, it can be conjugated into the RNA nanoparticle structure before in vitro transcription or chemical synthesis of RNA. Dr. Guo and his colleagues have successfully conjugated malachite green aptamer to RNA nanoparticles characterized by a 3WJ pRNA motif. The in vitro experiment indicated that the aptamer is still functional after conjugation into 3WJ-pRNA nanoparticles

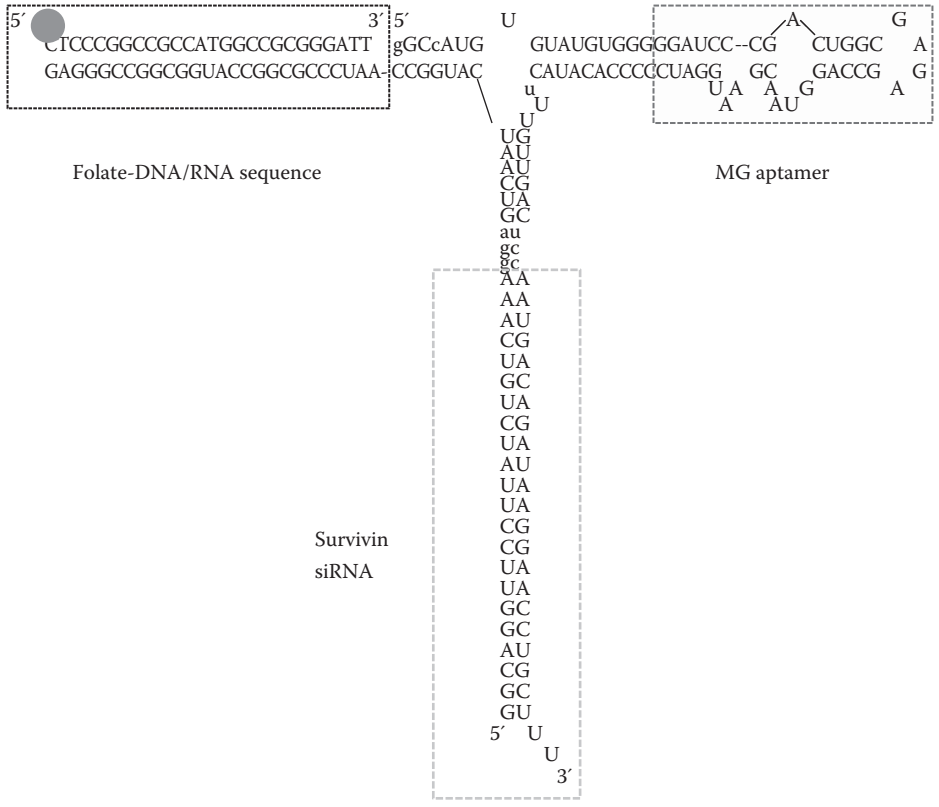

FIGURE 19.5
Diagram of RNA nanoparticle harboring malachite green aptamer, survivin siRNA and folate-DNA/RNA sequence for targeting delivery, using 3WJ-pRNA as scaffolds. (Reprinted by permission from Macmillan Publishers Ltd. *Nat. Nanotechnol*, Shu, D. et al., Thermodynamically stable RNA three-way junction for constructing multifunctional nanoparticles for delivery of therapeutics, 6, 658–667, copyright 2011.)

(Figure 19.5; Shu et al. 2011). The sequence for the malachite green aptamer nanoparticle was rationally designed with sequences of three pieces of 3WJ-pRNA motif. These three pieces of RNA strands were synthesized in vitro by transcription from a DNA template with T7 RNA polymerase, and the RNA nanoparticles were then self-assembled when the three RNA pieces were mixed in equal molar ratios. If there is currently no known RNA aptamer available for the desired applications, another approach for the conjugation of aptamers to RNA nanoparticle is to conjugate random sequences to a defined RNA nanoparticle structure. SELEX experiments, as described previously, will then be carried out with this library of random sequence bearing RNA nanoparticles against target peptides, proteins, or cells. The DNA library with random sequences is transcribed into an RNA library, and then partition techniques, such as nitrocellulose partitioning, capillary electrophoresis partitioning, etc., can be utilized to separate the bound and unbound RNA. The bound RNA nanoparticles are further eluted out and used as a template for reverse transcription and PCR for the next round of SELEX experiments.

19.3.3 Key Factors for Conjugating Aptamer to RNA Nanoparticles

When designing the aptamer-conjugated RNA nanoparticles, one key factor that needs to be considered is that the aptamer should be conjugated to the outsphere site of RNA nanoparticles, thus the aptamer-targeting delivery functionality can be achieved. Another key factor is to ensure that the aptamer will still fold correctly after conjugation to RNA nanoparticle nucleotides. If the aptamer has a double strand helix RNA end in its structure, we can connect both ends of aptamer to the open helix ends of RNA nanoparticles. If the aptamer has a single strand RNA loop end in its structure, then we can link only one end of the aptamer to the open ends of RNA nanoparticle carriers. For example, when designing anti-gp120 aptamer–conjugated pRNA nanoparticles, Zhou et al. designed two different chimeric pRNA/anti-gp120 aptamer constructs. In one structure, both the double strand helix ends of anti-gp120 aptamer were linked to the pRNA end bases of 23 to 97 (pRNA-A1-D3); in another structure, the anti-gp120 aptamer was directly appended to the 5′-end of pRNA (pRNA-A1-D4; Figure 19.6; Zhou et al. 2011). In vitro studies demonstrated that

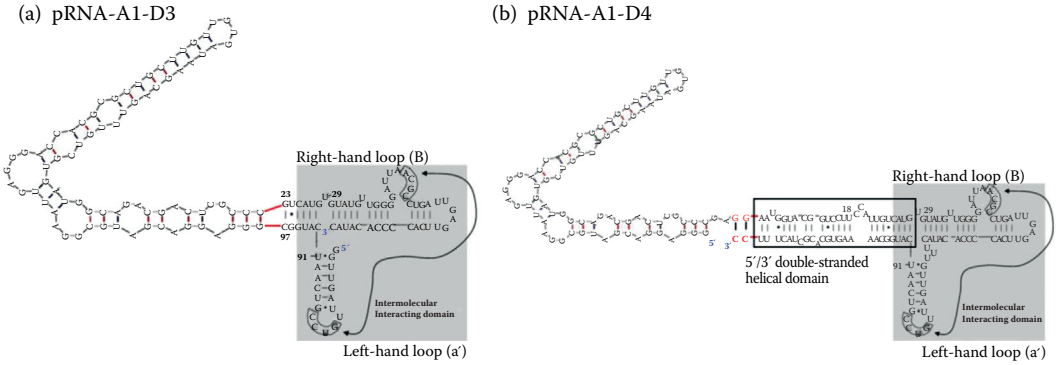

FIGURE 19.6
Schematic of pRNA nanoparticle harboring anti-HIV gp120 aptamer. (a) pRNA–A1-D3, the aptamer sequence was inserted into the 3′/5′ double helical domain (23 nt fragment) and loop domain (97 nt fragment). (b) pRNA-A1-D4, the aptamer sequence was directly appended to the 5′ end of pRNA 5′/3′ double-stranded helical domain. (Reprinted from *Methods*, 54, Zhou et al., Dual functional RNA nanoparticles containing phi29 motor pRNA and anti-gp120 aptamer for cell-type specific delivery and HIV-1 inhibition, 284–294, Copyright 2011, with permission from Elsevier.)

both pRNA–aptamer chimeras can specifically bind to and become internalized into cells expressing human immunodeficiency virus (HIV) gp120 with the dissociation constant (K_d) being approximately 48 nM for the pRNA-A1-D3, whereas it is approximately 79 nM for the pRNA-A1-D4.

19.4 Application Status of RNA Aptamer-Conjugated pRNA Nanoparticles

Because pRNA nanoparticles are composed entirely of RNA, conjugating RNA aptamers to pRNA to form a targeted delivery system will allow all the advantages of RNAs as therapeutic agents to be retained. We have described the structure and synthesis of both pRNA and the aptamers, and discussed the approaches and key factors in the designing of RNA aptamer-conjugated pRNA. Another key obstacle in applying the RNA aptamer–pRNA system for targeted therapy is that RNA is not stable and is susceptible to quick degradation in vivo in the bloodstream. Recently, researchers from Dr. Guo's laboratory found that 2′-F modification of pRNAs are both chemically and metabolically stable in vivo in animals. Importantly, they have shown that pRNA's function and biological activity stays intact, despite this 2′-F modification. To date, this pRNA nanoparticle delivery system has been used to conjugate CD4 aptamers and anti-gp120 aptamers, and was tested in anticancer and viral infection therapies.

Dr. Guo's laboratory has used the pRNA dimer nanoparticle to specifically deliver siRNA against the prosurvival gene called survivin to CD4-positive cells. This is accomplished by replacing the 3′/5′ double helix loop of the pRNA sequence with the survivin-silencing siRNA and by conjugating an anti-CD4 aptamer to the pRNA. They found that this dimer is able to specifically target CD4-positive lymphocytes to silence the target gene expression and reduce cell viability (Guo et al. 2005). Most recently, researchers from Dr. Guo's laboratory have discovered that the 3WJ of pRNA discussed above is the most stable structure found among the 25 3WJ motifs obtained from different biological systems (Shu et al. 2011). They have shown that each arm of the 3WJ-pRNA can carry the abovementioned CD4 receptor–binding RNA aptamer, siRNA, or ribozyme, and bring them into target cells both in vitro and in vivo. Importantly, they have further gone on to show that the 2′-F RNase-resistant form of 3WJ-pRNA also retains its folding and can carry these incorporated functional moieties to target cells both in vitro and in vivo.

Additionally, anti-gp120 aptamers have also been conjugated with the pRNA system by Dr. Rossi's group in their research against HIV-1 infections (Zhou et al. 2008, 2009, 2011; Zhou and Rossi 2011). The HIV-1 virus express a surface protein called glycoprotein gp120, which recognizes the CD4 cell receptor on the host's cells and initiates the membrane fusion that leads to subsequent delivery of viral RNA and enzymes. Once infected by HIV-1, these cells will then also express gp120 on their cell surface. Zhou et al. have previously generated gp120 aptamer chimeras with siRNA targeting the HIV-1 tat/rev gene region and found that these chimeras can be specifically internalized into cells expressing gp120 to silence the expression of target gene. Most recently, Dr. Rossi's group further used the pRNA system developed by Dr. Guo to generate dual functional RNA nanoparticle chimeras with anti-gp120 aptamers, as described previously, and achieved both cell type–specific delivery and targeted inhibition of viral replications (Zhou et al. 2011).

19.5 Conclusions and Future Perspective

The high affinity and specificity of RNA aptamers rivaling those of antibodies makes them a promising tool for targeted delivery of therapeutics. As discussed in this chapter, conjugating RNA aptamers to pRNA nanoparticles for targeted therapy has shown great promise in the treatment of cancer and viral infections. In addition to the abovementioned pRNA nanodelivery system, there have also been developments in other approaches for delivering therapeutics using RNA aptamers for disease treatments. With their many advantages as a key component of RNA nanotechnology, including its small size, high stability, multiconjugation capability, and especially its nonimmunogenic nature, RNA aptamers will no doubt find more applications in the targeted therapy arena, especially with more and more RNA aptamers isolated against an ever-increasing repertoire of disease targets. With strong interest and further development of RNA nanotechnology, and with the recent approval of RNA as a therapeutic by the Food and Drug Administration, we should also expect a bright future for RNA aptamers not only as a delivery tool for targeted therapy but also beyond.

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Small non-coding RNAs and aptamers in diagnostics and therapeutics

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Summary

Small non-coding RNAs (sncRNAs) such as small interference RNAs (siRNAs), microRNAs (miRNAs) and RNA aptamers have recently emerged as highly versatile and valuable tools in disease diagnostics and therapeutics, largely due to their key regulatory functions in many human diseases including cancer, viral infections, genetic disorders, etc. Recent technological advancements as described in the previous chapters have greatly aided the discovery of sncRNAs and their applications for disease detection and therapy. Here, we will describe the advantages of using sncRNAs as diagnostic and therapeutic tools, followed by some of the most recent examples of their use and a vision for the future perspective.

Key Words: Small Non-Coding RNA, siRNA, miRNA, RNA aptamer, RNA Nanotechnology, Diagnostics and Therapeutics

1.Introduction

The biological understanding of RNA function has dramatically expanded within the past thirty years with evidences supporting its key roles in protein synthesis, and more recently in gene expression regulation ***(1)***. The latter can, in part, be attributed to small non-coding RNAs (sncRNAs) such as small interfering RNAs (siRNAs) and micro RNAs (miRNAs) ***(2, 3)***. siRNAs are double stranded RNAs consisting of approximately 21 base pairs that have the ability to interfere with the expression of target genes by eliciting the RNA interference (RNAi) pathway. miRNAs are small single stranded RNAs of similar length that can recognize target mRNA transcripts to inhibit their translation into proteins. RNA aptamers are also considered sncRNAs because they are composed of single strands of RNA of various lengths but often less than 100-200 nucleotides. RNA aptamers are commonly selected through a process called SELEX for their ability to bind to a desired target, which can include proteins, cells, and other moieties with high affinity and specificity to regulate their function ***(4)***. The abundance of regulatory functions of small ncRNAs and aptamers in health and disease makes them especially valuable tools in diagnostics and therapeutics in clinical settings. In this chapter, we will discuss the advantages of using sncRNAs as therapeutic and diagnostic agents, followed by some of the most recent examples of their use in therapeutics and diagnostics and also a future perspective.

2. Advantages of Using RNA in Therapeutics and Diagnostics

There are multiple advantages to using small noncoding RNAs and RNA aptamers as therapeutic and diagnostic agents over other molecules commonly used in biomedicine

like antibodies and peptides. First, these noncoding RNAs are known to elicit little to no immunogenic effects, thus overcoming the most common major obstacle of current monoclonal antibody and peptide therapies ***(5)***. Secondly, small non-coding RNAs and RNA aptamers can be easily generated in high quantities and purity through controlled synthesis ***(6-8)***. In contrast, antibody preparation can be a very laborious and costly process that is often accompanied with low yields. Additionally, the thermodynamic stability of RNA is higher than that of peptides or antibodies, especially with the recent advancement of chemical (2'OMe, 2'F, 2'deoxy, 2'NH3) modifications that enable it to resist ribonuclease shearing and degradation *in vivo*. Along with increased thermodynamic stability, these chemical modifications can also enhance their pharmacokinetic and pharmacodynamic characteristics ***(9)***. From a structural standpoint, the single-stranded nature of RNA not only makes it easier to enter cells than DNA of the same nucleotide length but also allows for more specific and tighter binding to desired targets because of its smaller size and distinct tertiary folding structure. Furthermore, RNA can be conveniently conjugated to other functional molecules like ribozymes to create "riboswitches" or nanoparticles for tissue-specific and targeted delivery ***(9)***. The advantages addressed here highlight the versatility and capability of non-coding RNAs and RNA aptamers as tools in disease diagnostics and therapeutics, as evidenced by the most recent advancements made and examples described below.

3. sncRNA and RNA Aptamers in Disease Diagnostics

Traditionally, antibodies have been the primary diagnostic tool because of their ability to specifically target and bind to desired cell markers. With recent advancements in RNA

nanotechnology, small non-coding RNAs have evolved to be a novel, advantageous alternative. This section will focus on the diagnostic and prognostic applications of various small non-coding RNAs in human disease as imaging tools, disease cell detection devices and biomarkers, etc.

3.1 miRNAs as Diagnostic Tools

microRNAs (miRNAs) have recently emerged as highly promising biomarkers in the field of diagnostics as studies have demonstrated that relatively few miRNAs are able to provide large amounts of diagnostic and prognostic information detailing the specific disease and its progression ***(10, 11)***. For example, one study identified 4 miRNAs (hsa-miR-15b, hsa-miR-181b, hsa-miR-191 and hsa-miR-200c) that are significantly overexpressed in colorectal tumor samples in comparison to normal colorectal tissue, helping to support the use of miRNAs for early detection, screening and surveillance in colorectal and other occult cancers ***(12, 13)***. Moreover, a six-miRNA-based classifier using the LASSO Cox regression model has been used to effectively classify patients with stage II colon cancer into groups at low and high risk of disease recurrence, thereby adding prognostic value to the traditional clinicopathological risk factors ***(11)***. miRNAs can be further used as biomarkers for identifying tissue origin in metastatic tumors ***(13, 14)***. By measuring miRNA expression levels in 22 different tumor tissues and metastases, one group was able to classify the origins of two thirds of the samples with high confidence and greater than 90% accuracy ***(14)***. Excitingly, a panel of diagnostic miRNA biomarkers, like those mentioned here, are offered to clinicians to help identify

primary origins of tumor metastases where origins are unclear, helping to exemplify the true potential of miRNA diagnostics in clinical settings ***(15)***.

Circulating miRNA profiles from patient plasma and serum have also proven to be of important prognostic value. For example, miR-141, a microRNA highly overexpressed in prostate cancer, is readily measured in serum, and can be used to identify prostate cancer patients from non-cancer controls ***(16)***. More recently, it was reported that four miRNAs (miR-148b, miR-376c, miR-409-3p and miR-801) were significantly upregulated in the plasma of early stage breast cancer patients, implicating these miRNAs as potential biomarkers for early detection of breast cancer ***(17)***. In addition, miRNAs can be found in urine and saliva samples. It was observed that miR-125a and miR-200a reside in lower levels in patients' saliva with oral cancer than healthy individuals' saliva, thus highlighting the diagnostic potentials of miRNAs in cancer and disease detection through non-invasive approaches ***(18)***. In all, these circulating miRNA biomarkers represent a way for non-invasive clinical detection of cancer, and therefore make future diagnostic procedures less strenuous for both patient and clinician.

3.2 RNA Aptamers as Diagnostic Tools

As mentioned previously, RNA aptamers can recognize and bind to a desired target with high affinity and specificity, making aptamers an ideal tool for disease diagnosis as imaging devices. One such example includes a radiolabeled aptamer that can target and be quickly taken up by desired tumors. Tenascin C is an extracellular matrix protein that is over-expressed in multiple tumor types, including breast, lung, colon, prostate and

lymphoma. A radiolabeled anti tenascin-C aptamer (TTA1-^{99m}Tc) has been used to target such cell types, with efficient uptake due to rapid blood clearance and tumor penetration rates with a half-life of less than two minutes ***(19)***. As an advantage of this rapid uptake of aptamers by tumors, the bloodstream is quickly cleared of any unconsumed radioactive substances. In addition to high-quality tumor imaging, this method also limits toxicity that typically accompanies the use of radioactively labeled antibodies in response to their inability to be cleared quickly from the body ***(20)***. Another example of such application in tumor imaging is the A10 aptamer that binds specifically to PMSA, a prominent prostate cancer marker. When conjugated to quantum dots and a low-toxicity contrast agent typically used in MRI imaging known as TCL-SPION (thermally cross-linked supraparamagnetic iron oxide nanoparticle), the TCL-SPION-A10 aptamer allows for efficient imaging and recognition of prostate cancer tumors ***(21, 22)***. Additional examples of RNA aptamers characterized with diagnostic attributes include RNA aptamers that target EGFR (Epidermal Growth Factor Receptor), a receptor tyrosine kinase strongly overexpressed in more than half of Glioblastoma multiforme (GMB) tumors, CD30 of Hodgkin's Lymphoma, p68 RNA Helicase of intrhepatic CT26 tumors and colorectal tumor cells, and $hVEGF_{165}$ that is expressed in a variety of different cancer cell types ***(4)***. Utilization of these aptamers not only addresses early detection difficulties that arise in many cancer patients, but also provides a novel surgical verification method to confirm that local tissue margins of removed tumors are indeed now disease free.

4. sncRNAs and RNA Aptamers in Therapeutics

Due to their ability to target almost any given intra- and extracellular components of key signaling pathways involved in human diseases, sncRNAs and RNA aptamers have started to become highly attractive new-generation therapeutic agents to target these signaling pathways for the treatment of cancer, viral infections and other diseases ***(4, 23)***. Here, we will briefly overview the current advancements and provide several examples of applying small ncRNAs and RNA aptamers for targeted therapy. With the exciting FDA approval of an RNA aptamer-based therapy and, most recently, RNA antisense oligonucleotide-based therapeutics for clinical use, we expect this area of research will gain huge momentum with many more sncRNA- and RNA aptamer-based applications emerging in disease therapeutics in there very near future.

4.1 RNA Aptamers in Therapuetics

One of most significant therapeutic developments in the RNA aptamer field is the approval of Pegaptanib (Macugen, Eyetech Pharmaceutics/Pfizer) by the US Food and Drug Administration (FDA) for the treatment of age-related macular degeneration (AMD) in 2004 ***(24, 25)***. Pegaptanib is an RNA aptamer against Vascular Endothelial Growth Factor isoform 165 ($VEGF_{165}$), an important protein known to function in angiogenesis and neovascularization in age-related macular degeneration (AMD) and diabetic macular edema (DME) ***(24, 25)***. Significantly, this particular RNA aptamer, Pegaptanib, has exhibited superior ability to bind and block extracellular $VEGF_{165}$'s mitogenic function and vascular permeability enhancing ability. Remarkably, it elicits no toxic side effects and has a very long half-life with one study showing that it can remain biologically active in the eye for as long as 28 days***(24, 25)***.

Following the arrival of an FDA approved and clinically administered RNA aptamer-based drug, researchers began extensively seeking out other aptamers with high therapeutic potential, especially for cancer treatments. One group identified RNA aptamer E07 that has high binding affinity and specificity for EGFR. They found aptamer E07 can be readily taken up by EGFR-expressing cancer cells, indicating its potential as an EGFR-targeted therapeutic vehicle to escort other anti-cancer moieties into specific target cells ***(26)***. Another group conjugated an anti-prostate specific membrane antigen (PSMA) aptamer, A9, previously shown to tightly bind prostate tumor cells, with siRNAs targeting lamin A/C or GAPDH ***(27)***. This aptamer-siRNA conjugate showed successful knockdown of target gene expression in PSMA expressing cells but not in negative controls ***(27)***. Similar results were seen when RNA aptamer A10, also known to specifically target PSMA, was conjugated to siRNAs against polio-like kinase 1 (PLK1) and B-cell lymphoma 2 (BCL2) ***(28)***. These aptamer-siRNA conjugates have been further tested both in vitro and in vivo in animal models with very exciting data showing successful siRNA-mediated depletion of target proteins and death of prostate cancer cells ***(28)***.

Additionally, a siRNA-RNA aptamer conjugate is currently under development for therapies against infections of Human Immunodeficiency Virus type 1 (HIV-1). For the HIV-1 virus to replicate, it uses glycoprotein gp120 to recognize cell surface receptor CD4 in host cells to initiate membrane fusion for the delivery of viral material. By linking an anti-gp120 aptamer with anti-HIV tat/rev siRNAs, it was found that this

siRNA-RNA aptamer conjugate can be efficiently and specifically taken up by gp120-expressing cells ***(29)***. The linked anti-tat/rev siRNA was then processed by endoribonuclease Dicer, which led to successful inhibition HIV replication while eliciting no immune response ***(29)***. These advancements highlight a promising future for siRNA-RNA aptamer conjugates not only as therapeutic reagents for cancer, but also for the treatment of viral infection and other diseases.

4.2 miRNAs, siRNAs, and ASOs in Therapeutics

In a similar fashion to RNA aptamers, sncRNAs like miRNAs, siRNAs and antisense oligonucleotides (ASOs) have proven to be successful in the field of disease therapeutics. Indeed, an antisense oligonucleotide-based therapy (Kynamro) has recently gained FDA approval for the treatment of high cholesterol, a major contributor to Coronary Heart Disease (CDH). Kynamro is a so called "second generation" antisense oligonucleotide with aforementioned chemical modifications including 2' methoxyethyl substitutions at the 5' and 3' ends of the oligonucleotides and the addition of phosphorothioates at each internucleotide linkage that greatly increase the efficacy and stability of the moieties in serum ***(15)***. Once injected, these oligonucleotides can target apolipoprotein B100 mRNA transcripts for degradation and thus inhibit cholesterol production in the liver ***(15)***. The success of Kynamro highlighted how chemical modification, like those made to RNA aptamers, to sncRNAs can provide for stable and successful in vivo targeted therapies for human disease.

In addition to RNA aptamers and ASOs, siRNAs and miRNAs have shown strong potential in the field of disease therapeutics. There are numerous siRNA-based therapies currently in preclinical development, with some of such siRNAs in early-stage clinical trials for treatment of diseases including pachyonchyia congenita, menopausal osteoporosis, and pancreatic and other advanced cancers. As previously described of aptamers and other small ncRNAs, successful delivery is the key for the effectiveness of siRNA-mediated downregulation of specific target genes. Recent progress has been made to increase the potential for successful siRNA-delivery by utilizing modified lipid-like carriers, siRNA conjugates, as well as targeted nanoparticles that can stabilize and aid in the specific targeted delivery of siRNAs ***(30, 31)***. One such example is the Triantennary GalNAc- siRNA conjugate, which has 3 GalNAc molecules attached to the 3' terminus of the siRNA thorugh a triantennary spacer ***(32)***. The most advanced GalNAc-siRNA is the ALN-TTRsc, which silences transthyretin (TTR) in attempt to treat TTR-amyloidosis, has recently entered phase 1 clinical trials with high potential for success ***(32)***. Another example of siRNA demonstrating successful therapeutic potential is a lipid nanoparticle-siRNA conjugate targeting VEGF and kinesin spindle protein (KSP) for cancer treatment ***(33)***. In recent in-human trials, this siRNA conjugate produced a multitude of desired effects, among them the complete regression of liver metastases in endometrial cancer ***(33)***. With the continued optimization of siRNA delivery methods, siRNA-based therapeutics will continue to expand in the field of disease therapeutics, with a continued trend in clinical trial entrance and approval for clinical use expected in the near future ***(34, 35)***.

miRNAs have the unique characteristic to serve as both therapeutic agents and therapeutic targets for disease treatments ***(9)***. The latter is characterized in part by the use of modified antisense-miRNA oligonucleotides, termed "anti-miRs" ***(15)***. For example, Miravirsen is a modified oligonucleotide designed to inhibit miR-122, a miRNA which is necessary for functional infection of the Hepatitus C Virus (HCV). Presently, Miravirsen has completed two phase I clinical trials and is currently enrolled in a phase IIa clinical trial showing promising results in significantly lower levels of HCV RNA in HCV patients ***(15)***. As therapeutic agents, re-expression of miRNAs has also provided multiple pre-clinical evidences of being successful for disease therapy. For example, Chronic Lymphocytic Leukemia (CLL) is a prominent human leukemia characterized by malignant B cells that overexpress apoptotic BCL2 protein, while microRNAs miR-15a and miR-16-1 levels have shown to be inversely correlated with BCL2 in CLL tumor growth ***(36)***. It was found that re-introduction of miR-15a and miR-16-1 expression could lead to reduced levels of BCL2 and suppression of tumor growth. In another example, miR-4423 was known to be down-regulated in most lung tumors and its etopic expression has been shown to significantly inhibit the lung tumor growth ***(37)***. Currently, there are increasing examples of pre-clinical miRNAs with the therapeutic potential similar to what we have outlined above ***(9)***. With the development of efficient approaches to deliver miRNA mimics and anti-miRs, and additional *in vivo* and clinical testing, we expect they will likely to make important impacts on the treatment of many diseases.

5. Conclusion and Future Perspective

In this chapter, we have discussed the characteristics of sncRNAs and how they can be used as advantageous diagnostic and therapeutic agents. We have also provided some success stories and future examples of the use of sncRNAs as novel therapeutics for human diseases, biomarkers and early detectors of cancer types, identifiers of metastatic tumor tissue origins and inhibitors of viral transmission etc. As discussed in the preceding chapters of this book, recent advancements in technologies such as next generation sequencing, zinc-mediated RNA fragmentation, RNase protection assays, new in vitro and in vivo cell and tissue-based SELEX procedures etc., have aided in the identification of sncRNAs. Once identified, sncRNAs can be further visualized and analyzed using a number of different procedures, including *in situ* hybridization, microarray and quantitative proteomics. We expect these new technical advancements will greatly increase the repertoire of sncRNAs and broaden their applications in the diagnosis and therapy of human diseases. With the FDA approval of the first RNA aptamer-based drug therapy almost a decade ago and the most recent second-generation antisense RNA treatment last year, there is no doubt that this field will gain sustained momentum with an explosion in the discovery of new sncRNAs for novel diagnostic and therapeutic applications. Combined with the many advantages that sncRNAs have over other traditional reagents such as antibodies, we have a plethora of reasons to be fully optimistic about the future of sncRNAs as a mainstay of next generation medicine in disease diagnostics and therapeutics.

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